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Development of a Resistance-like Phenotype to Sorafenib by Human Hepatocellular Carcinoma Cells Is Reversible and Can Be Delayed by Metronomic UFT Chemotherapy[1]

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Abstract

Acquired resistance to antiangiogenic drugs, such as sorafenib, is a major clinical problem. We studied development of a resistance to sorafenib in new preclinical models of human hepatocellular carcinoma (HCC) along with a strategy to delay such resistance—combination with metronomic chemotherapy. Three different xenograft models were studied using human Hep3B HCC cells, which are highly responsive to sorafenib, namely, orthotopic and subcutaneous transplant in severe combined immunodeficient mice, and an orthotopic transplant in nude mice. The complementary DNA for the β-subunit of human choriogonadotropin was transfected into HCC cells, and urine levels of the protein were monitored as a surrogate of tumor burden. Extended daily treatments, sometimes interrupted by a break period of 3 to 7 days to allow recovery from toxicity at sorafenib doses of 30 to 60 mg/kg, were maintained until and after evidence of tumor relapse. Initially responsive tumors seemed to develop a resistance-like phenotype after long-term daily treatment (e.g., >42 days) at doses of 30 to 60 mg/kg. Transplantation of cell lines established from progressing tumors into new hosts showed that the resistant phenotype was not propagated. Furthermore, a regimen of daily metronomic uracil + tegafur (UFT, an oral 5-fluorouracil prodrug) chemotherapy with a less toxic regimen of sorafenib (15 mg/kg per day) significantly delayed the onset of resistance (>91 days). In conclusion, development of a resistance-like phenotype to sorafenib is reversible, and metronomic UFT plus sorafenib may be a promising and well-tolerated treatment for increasing efficacy by delaying emergence of such resistance.

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Introduction

The increasing and widespread clinical use of antiangiogenic drugs has revealed that both intrinsic and acquired resistance to this new clinical class of anticancer agent are significant problems that limit their efficacy [1–3], similar to other classes of anticancer drug such as chemotherapy, hormone or hormone receptor targeting therapies, and oncogene-targeting signal transduction inhibitors. For example, the progression-free survival or overall survival benefits induced by bevacizumab, the anti–vascular endothelial growth factor (VEGF) monoclonal antibody, when combined with chemotherapy are modest, that is, in the range of 1 month to several months, based on randomized phase 3 clinical trial results in metastatic colorectal, breast, and non–small cell lung cancer [3]. Small-molecule tyrosine kinase inhibitors (TKIs) that target VEGF and platelet-derived growth factor (PDGF) receptors, among others, provide similar benefits as monotherapies for renal cell carcinoma and hepatocellular carcinoma [2]. These results have stimulated some empirical strategies to delay the onset of apparent acquired resistance or to treat cancers that have progressed on antiangiogenic therapy [2]. An example of such approaches includes sequential therapy with different antiangiogenic drugs [4–6]. For example, renal cell cancer (RCC) patients whose tumors initially respond to sorafenib but

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later start progressing can sometimes respond again when treated with a similar, but not identical, VEGF pathway–targeting drug, for example, sunitinib [4,5]. A number of such sequential therapy approaches are under investigation in the clinic [2,4].

Surprisingly, only a few preclinical studies have been reported thus far dealing with the topic of acquired resistance to antiangiogenic drugs, despite the obvious growing clinical importance of the phenomenon. Such studies could clearly provide new insights into the mechanisms underlying such resistance and thus strategies that might be considered for either circumventing or delaying such resistance. The studies that have been published suggest a number of possible, and diverse, mechanisms of resistance such as "evasive resistance" whereby compensatory proangiogenic pathways are induced or upregulated in tumor cells, such as basic fibroblast growth factor–mediated tumor angiogenesis during therapy with a drug that specifically targets the VEGF pathway, for example, anti–VEGFR-2 antibodies [7]; this may occur as a consequence of increased tumor hypoxia induced by the antiangiogenic drug treatment [7,8]. If so, strategies to target the (elevated) hypoxia-inducible factor 1α accompanying the increased hypoxia could represent a promising therapeutic approach to delay the resistant phenotype, for example, by using metronomic chemotherapy regimens that target hypoxia-inducible factor 1α [8–10]. Other suggested mechanisms of acquired resistance include selection of tumor cell variants that can survive under hypoxic conditions [11], rapid vascular remodeling resulting in more mature vessels that are not responsive to antiangiogenic drugs [12,13], and production of alternate proangiogenic growth factors such as PDGF-C by cells in the stroma such as fibroblasts rather than the tumor cells themselves [14]. With respect to intrinsic resistance to a VEGF inhibitor, mobilization and tumor homing of proangiogenic bone marrow–derived myeloid-type cells, which express CD11b, and Gr1 has been proposed as a contributory mechanism [15], and as such, could be involved in acquired resistance as well.

Interestingly, all of the aforementioned preclinical studies were undertaken using protein-based drugs such as antibodies directed to VEGFR-2 [7,11], or to VEGF itself, either using soluble VEGF receptor trap/decoy drugs or anti-VEGF antibodies [14]. We are aware of two very recent studies dealing with acquired resistance to oral small molecule VEGF receptor multitargeting antiangiogenic TKIs, one by Hammers et al. [16] and the other by Huang et al. [17]. Both deal with sunitinib. In the former study, acquired resistance to sunitinib using a preclinical model of human renal cell carcinoma xenografts was found to be due, at least in part, to induction of interleukin 8–mediated tumor angiogenesis [17]. In this case, the resistant phenotype seemed to be stable and heritable [17]. In contrast, the study by Hammers et al. [16], using renal cell carcinoma fragments from patients relapsing on sunitinib therapy that were transplanted into immune-deficient mice, showed that the resistant phenotype was unstable and was associated with a reversible epithelial-to-mesenchymal phenotype.

The major purpose of this study was to develop an orthotopic preclinical model of acquired resistance or a resistance-like phenotype to the small molecule antiangiogenic TKI, sorafenib, in advanced human hepatocellular carcinoma (HCC). Sorafenib, which targets all three VEGF receptors and PDGF-B receptors, as well as some others, for example, c-kit, flt3, ret, and raf kinases, has shown transient but statistically significant clinical benefits in advanced HCC in two large randomized phase 3 trials [18,19] and is now approved for treatment of this disease. To develop a model of development of sorafenib resistance, we used three important procedures. First, we used a human HCC line that is initially highly responsive to the drug, even when orthotopically growing in the liver and treatment is initiated later at a more advanced (established) stage of growth, rather than immediately after transplantation of the cells [20]. Second, and perhaps most importantly, extended and effective treatments were maintained until evidence of onset of a more rapid progressive tumor growth ("relapse") was observed, indicative of the possible development of resistance to the drug. Third, we monitored tumor response in the liver, and even subcutaneously, by a molecular surrogate biomarker approach, namely measuring a protein secreted from the HCC cells—the β-subunit of human choriogonadotropin (β-hCG), detected in the urine [21]. This was made possible by transfecting a complementary DNA (cDNA) for β-hCG into the HCC cells as previously described in other model systems [21], similar to other studies using other human cell lines [22,23].

Here we report that cells of sorafenib-sensitive human HCC tumor xenografts progressing on sorafenib after approximately 1 month or more of daily treatment do not express the phenotype in a heritable manner because it was completely lost when the cells were transplanted to new hosts, which were then treated with sorafenib. We also report that concurrent therapy using a nontoxic metronomic chemotherapy regimen with the drug UFT (uracil + tegafur), a 5-fluorouracil (5-FU) oral prodrug [24], can significantly delay the onset of resistance to sorafenib in the original (autochthonous) host, and do so without increasing overt toxicity. The implications that some forms of a resistant-like phenotype developing to a drug such as sorafenib not being stable are discussed.

Materials and Methods

Cell Lines

The human HCC cell line Hep3B was purchased from ATCC (Manassas, VA). They were maintained in Dulbecco's modified Eagle medium (DMEM) with high glucose (Hyclone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), at 37°C in a humidified atmosphere containing 5% CO_2. Hep3B cells were transfected with the hCG.pIRES vector, and hCG-expressing variants were obtained by puromycin selection. The hCG.pIRES vector carries the cDNA for β-subunit of human choriogonadotropin (β-hCG), and the secreted protein can be detected in the urine as a surrogate marker for tumor burden [21]. The Hep3B-hCG clone was used for the experiment based on the highest levels of protein expression. Cells were dissociated by trypsin, washed once in serum-containing DMEM followed by two washes in serum-free DMEM, and then resuspended in serum-free DMEM for implantation.

Animals

Female CB17 severe combined immunodeficient (SCID; Charles River Canada, Quebec, Canada) or athymic nude (*nu/nu*) mice (Harlan, Indianapolis, IN) aged between 6 and 8 weeks were used. All animals were housed in microisolator cages, and procedures were carried out in accordance with institutional guidelines for proper animal care and maintenance.

β-hCG Measurements

Urine β-hCG was measured with the commercially available Pathozyme Free β-hCG ELISA Kit (Omega Diagnostics Ltd, Scotland, UK), which allows for quantitative determination of β-hCG. Urine β-hCG levels were normalized by concomitant measurement of urine creatinine levels (using QuantiChrom Creatinine assay kit from BioAssay Systems,

Hayward, CA) as described previously by Shih et al. [21]. Urine was collected by placing mice individually into small sterile aerated boxes for 2 hours.

Subcutaneous (Ectopic) Transplantation of Human HCC Cells

A bolus of five million Hep3B-hCG cells was injected subcutaneously into CB-17 SCID mice. Tumor size was assessed weekly by means of Vernier calipers and the formula $(w_1 \times w_2 \times w_2) / 2$, where w_1 and w_2 are the length and width (mm), respectively.

Orthotopic (Intrahepatic) Implantation of HCC Cells

Aseptic technique was used throughout the surgical procedure [20]. The anesthetized mouse was laid on its back, and a 1-cm transverse incision was made through the skin and peritoneum of the left upper abdomen. A portion of the liver was exposed by applying gentle pressure on the abdomen. We injected 10^6 cells in a 10-μl volume into the subsera of the liver by using a 25-μl Hamilton syringe and 30-gauge needle. After swabbing the area with sterile gauze, the pressure was removed from the abdomen, allowing the liver to slip back into place. The peritoneum was closed with 4-0 absorbable suture, and the skin was closed with wound clips.

Drugs and Treatment Schedules Used

The antiangiogenic drug sorafenib tosylate, an oral multitargeting receptor TKI, was generously provided by Bayer (Leverkusen, Germany), with the assistance of Dr. Scott Wilhelm. Known targets include all three VEGF receptors, PDGF-B receptors, and B-Raf, c-KIT receptor, and ret [25]. Sorafenib was prepared fresh daily just before gavaging, by dissolving in cremorphor EL/95% ethanol/water (12.5:12.5:75) as described previously [26,27]. The drug was administered by daily gavage at dose levels of 15, 30, and 60 mg/kg body weight starting between days 12 and 14 when all animals in the study had evidence of established tumors averaging from 100 to 150 mg based on β-hCG values, with five mice per group.

For the metronomic chemotherapy treatments, UFT, an oral 5-FU prodrug, which consists of a 4:1 molar combination of uracil (U) and tegafur (FT) was used in 0.1% hydroxypropylmethyl cellulose [28,29]. UFT is a first-generation dihydropyrimidine dehydrogenase inhibitory flouropyrimidine drug. Tegafur, uracil, and the hydroxypropylmethyl cellulose were generously supplied by Taiho Pharmaceutical Co, Ltd (Tokyo, Japan) with the assistance of Dr. Teiji Takechi. UFT was prepared fresh daily just before gavaging. The dosage of UFT used was 15/mg/kg per day administered by gavage. This was previously assessed as an optimal biologic dose of the drug when given in a daily nonstop metronomic fashion [24]. Cyclophosphamide (CTX; Baxter Oncology GmbH, Mississauga, Ontario, Canada) was purchased from the institutional pharmacy. CTX was reconstituted as per manufacturer's instructions to a stock concentration of 20 mg/ml and administered through drinking water to provide an estimated dosage of 20 mg/kg per day of CTX based on the estimated daily consumption of 3 ml for a 20-g mouse, as previously described [30].

Statistical Analysis

Results are reported as the mean ± SD. Statistical significance of differences in the sorafenib therapy experiments was assessed by two-way analysis of variance. Survival curves were generated by the Kaplan-Meier method, and the statistical significance of differences in survival was assessed by χ^2. All statistics was generated by using GraphPad Prism 4.00 software (GraphPad Software Inc., San Diego, CA). The level of significance was set at $P < .05$. Statistical analyses were based on the overall trend of the growth curves.

Results

Effect of Sorafenib Treatment on Tumors Derived from HCC Cells Orthotopically Injected into SCID Mice

We developed an orthotopic model of HCC by implanting human Hep3B cells in the livers of SCID mice as described in the Materials and Methods and elsewhere [20]. To help monitor disease burden and progression, the Hep3B cells were transfected with β-hCG cDNA, which results in secretable protein that can be measured in urine as a biomarker of tumor burden [20–23]. Next, we used this model to test the antitumor effect of different sorafenib monotherapies, as well as combinations of sorafenib with metronomic chemotherapy (Figure 1*A*). Three different doses of sorafenib were used for single-agent treatment, a decision that was guided by previously published preclinical studies reported by others [25–27,31]. We found sorafenib alone administered at 60 mg/kg per day (Figure 1*A*) was toxic to SCID mice because it caused a 10% body weight loss within the first 4 weeks of treatment (Figure 1*C*). Treatment was therefore suspended and was subsequently resumed only after the body weights of mice returned to the levels measured before treatment was begun. Lower doses of sorafenib that we tested also caused toxicity. However, this became evident only after much longer periods of treatment. Thus, sorafenib at 30 mg/kg per day caused a 10% weight loss after 38 days of treatment (Figure 1*C*), whereas using the 15-mg/kg per day regimen, this toxicity became evident only after 56 days of daily treatment. The combination of sorafenib (30 mg/kg per day) with metronomic oral CTX administered through the drinking water at a dose of 20 mg/kg per day caused a 10% weight loss within 10 days of treatment, whereas for the combination of sorafenib (30 mg/kg per day) with metronomic UFT (15 mg/kg per day), weight loss of a similar magnitude became significant after 38 days of treatment. In previous studies, neither metronomic UFT nor CTX, nor concurrent combination of the two drugs, caused significant weight loss, even after more than 140 days of continuous daily treatment of the combination [24]. Thus, whenever significant weight loss was observed, only the administration of sorafenib was suspended (so that in the combination therapy groups, the administration of the metronomic CTX or UFT was continued), and sorafenib treatment was resumed when the mouse body weights recovered (Figure 1*C*); these sorafenib drug "holidays" ranged from 3 to 9 days in duration.

Preclinical Effect of Sorafenib Therapies on Orthotopic HCC Growth as Assessed by Urine β-hCG Levels

Despite the difficulties that arose because of the toxicity associated with sorafenib treatment in SCID mice, the urine β-hCG data suggested a significant antitumor effect of the various regimens we tested. Thus, after 1 week of daily treatment, we observed marked differences in the β-hCG urine levels in the different groups (Figure 1*A*). The control group's β-hCG levels rose rapidly until day 35 (at which point, some of the mice became moribund). In contrast, all the treatment groups showed a delay in the increase of urine β-hCG levels. The highest β-hCG levels within the sorafenib treatment groups were seen with sorafenib monotherapy using the lowest dosage, that is, 15 mg/kg per day (Figure 1*A*), whereas the lowest β-hCG levels were observed using

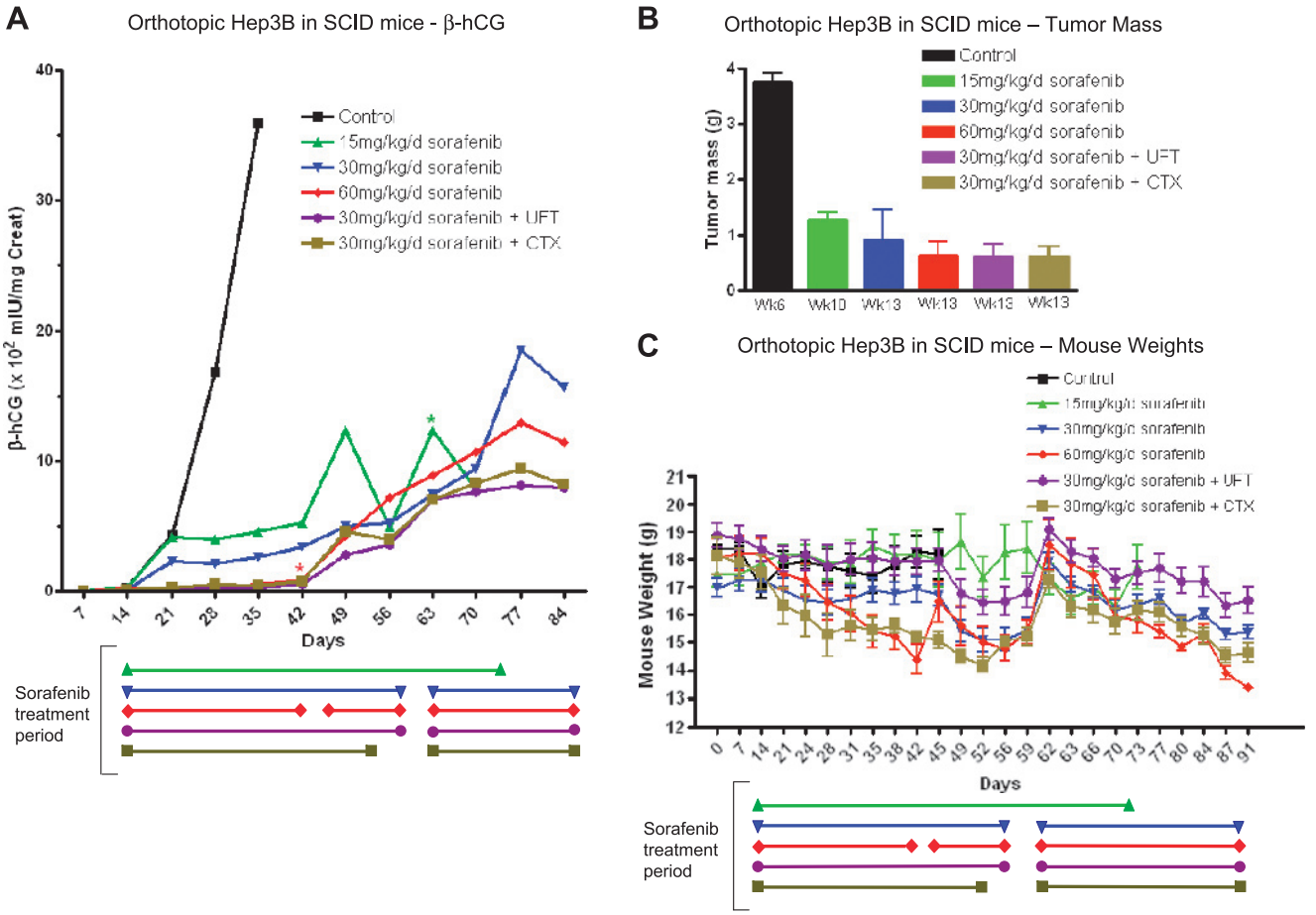


Figure 1. Effect of sorafenib regimens on orthotopically implanted human Hep3B HCC cells in SCID mice. Hep3B cells were tagged with β-hCG and implanted into the left lobe of the liver ($n = 5$ per treatment group). (A) Urine β-hCG levels of pooled samples, normalized to urine creatinine levels, of mice treated with sorafenib monotherapies (at 15, 30, or 60 mg/kg per day by gavage) or sorafenib (at 30 mg/kg per day) combined with either metronomic CTX (20 mg/kg per day, per os) or metronomic UFT (15 mg/kg per day by gavage). Treatments were carried out during the periods indicated with breaks in sorafenib dosing starting when toxicity (i.e., >10% average body weight loss) was observed in each treatment group. The graph shows the beneficial effect of the sorafenib regimens compared with controls treated by gavage of vehicle alone, on the relative tumor burden. Green asterisk indicates a mouse treated with 15 mg/kg per day of sorafenib that died at that time point. Red asterisk indicates a mouse treated with 60 mg/kg per day of sorafenib that died at that time point. (B) Corresponding weight measurements of the tumor mass in the liver of mice in each treatment group at the time of sacrifice (note that because of the different treatment efficacies, mice from the groups were killed at different weeks after the initiation of treatment, as indicated). (C) Corresponding mouse weight curves as a measure of relative toxicity of the different sorafenib treatment regimens. Note that for individual groups, sorafenib treatment was suspended for the indicated times whenever toxicity was observed in each group.

the following regimens: 1) 60 mg/kg per day sorafenib, 2) 30 mg/kg per day sorafenib plus UFT, and 3) 30 mg/kg per day sorafenib plus CTX. The sudden drop in β-hCG levels in the 15-mg/kg per day sorafenib group on day 56 (Figure 1*A*) coincided with the death of one mouse in this group before urine collection. Similarly, one mouse died in the control group on day 42, and the remaining mice became moribund 3 days later; the control group was therefore terminated on day 45 (i.e., 3 weeks after treatment initiation). For the 15-mg/kg per day sorafenib monotherapy group, all the remaining mice also began to show weight loss or manifested a moribund state by day 73, at which point mice from this group were also killed. However, all the other treatment groups did not show signs of advanced disease until day 84 (i.e., week 10 after treatment initiation), at which point the experiment was terminated, and mice from all groups were killed. The mean weights of tumor mass in the livers of the mice at the time of sacrifice (Figure 1*B*) were consistent with the β-hCG data. Thus, they showed that the sorafenib treatment regimens caused a reduction in the tumor burden compared with controls. Taken together, these results suggest that the sorafenib regimens are effective in treating orthotopically implanted established Hep3B tumors, although the prolonged sorafenib treatment regimens using the higher doses are associated with significant host toxicity in SCID mice.

Effect of Sorafenib Treatment on Subcutaneous HCC Tumors in SCID Mice

Hep3B cells were also injected subcutaneously in SCID mice to further evaluate the effect of sorafenib treatment in this more conventional model of therapy testing (Figure 2*A*). Furthermore, caliper measurements of the subcutaneously growing tumors in this experiment allowed us to confirm the reliability of the β-hCG marker as a readout for relative tumor burden (Figure 2*B*). Similar to our observations using the orthotopic tumor transplant model, we again noted that the sorafenib monotherapies we tested (at 15, 30, or 60 mg/kg per

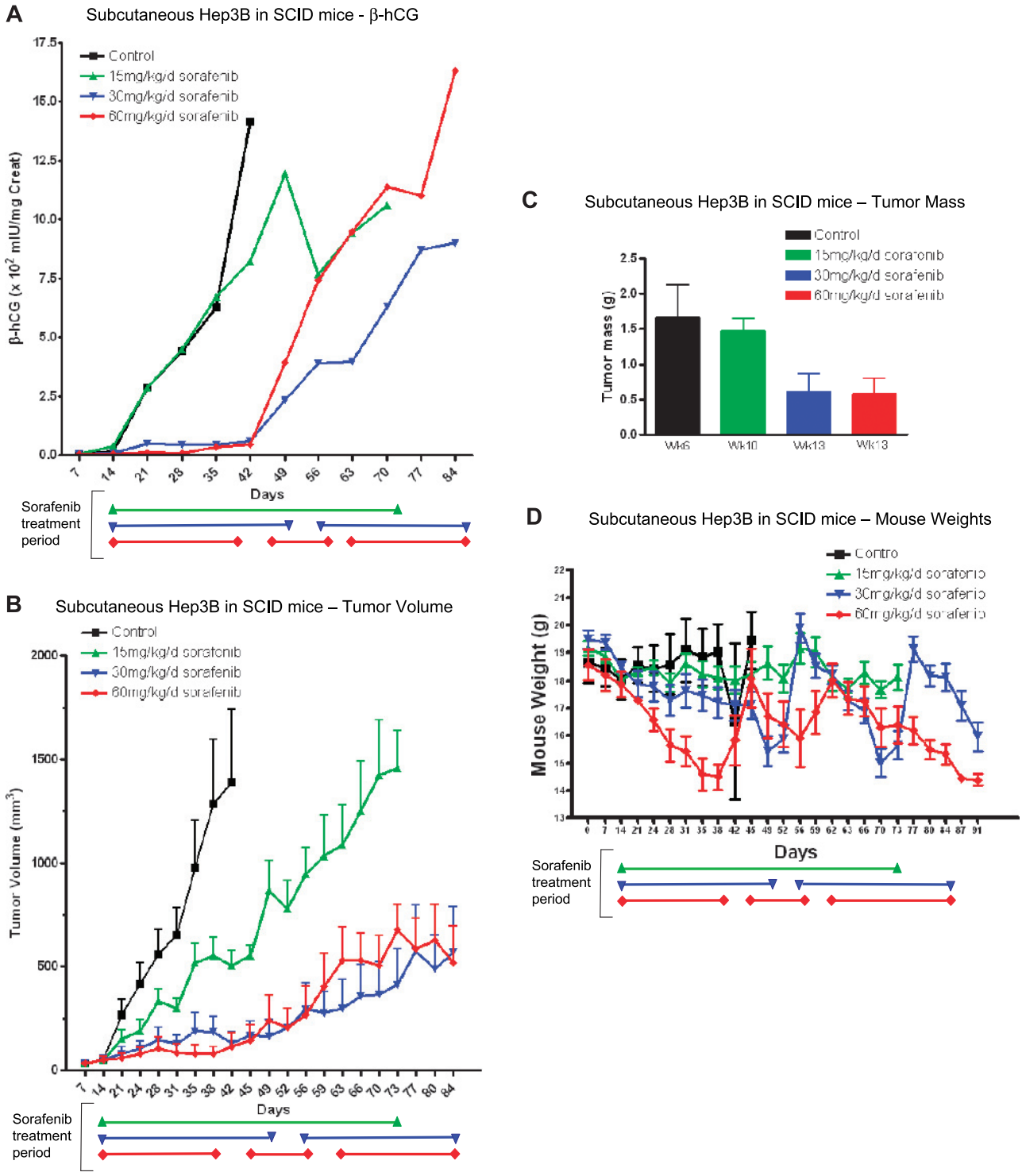


Figure 2. Effect of sorafenib regimens on subcutaneously implanted Hep3B cells in SCID mice. Hep3B cells were tagged with β-hCG and implanted into the left flank of SCID mice ($n = 5$ per treatment group). (A) Urine β-hCG levels of pooled samples, normalized to urine creatinine levels, of mice treated with sorafenib monotherapies (at 15, 30, or 60 mg/kg per day by gavage). Treatments were carried out during the periods indicated with breaks in sorafenib dosing starting when toxicity (i.e., >10% average body weight loss) was observed in each treatment group. The graph shows the beneficial effect of the sorafenib regimens, compared with controls treated by gavage of vehicle alone, on the relative tumor burden. (B) Corresponding tumor volume curves mirror the beneficial effect of the sorafenib regimens compared with controls. (C) Corresponding weight measurements of the tumor mass of mice in each treatment group at the time of sacrifice (note that because of the different treatment efficacies, mice from the groups were killed at different weeks after the initiation of treatment, as indicated). (D) Corresponding mouse weight curves as a measure of relative toxicity of the different sorafenib regimens. Note that for individual groups, sorafenib treatment was suspended for the indicated times whenever toxicity was observed in each group.

day) significantly inhibited tumor growth compared with vehicle-treated controls (Figure 2*A*). The β-hCG curves obtained were found to be in good agreement with the more traditional tumor caliper measurements (Figure 2*B*), as well as with the mean weight of the tumor mass in the mouse livers when the experiment was terminated, as shown in Figure 2*C*. Note that because of the different treatment efficacies, mice from the various groups were killed at different times after the start of treatment, as indicated. However, once again, we found that the antitumor benefit of the sorafenib treatments came at the expense of toxicity, which emerged after prolonged drug administration schedules (Figure 2*D*, with the exception of the 15-mg/kg per day treatment group). Because of this treatment-associated weight loss, we again had to interrupt sorafenib administration for periods ranging from 5 to 7 days (Figure 2*D*) in the 30- or 60-mg/kg per day treatment group. In conclusion, sorafenib is effective for the treatment of established primary Hep3B tumors in SCID mice, but the treatment-associated toxicity in such mice necessitated occasional short breaks in therapy. Also notable are the marked antitumor effects observed,

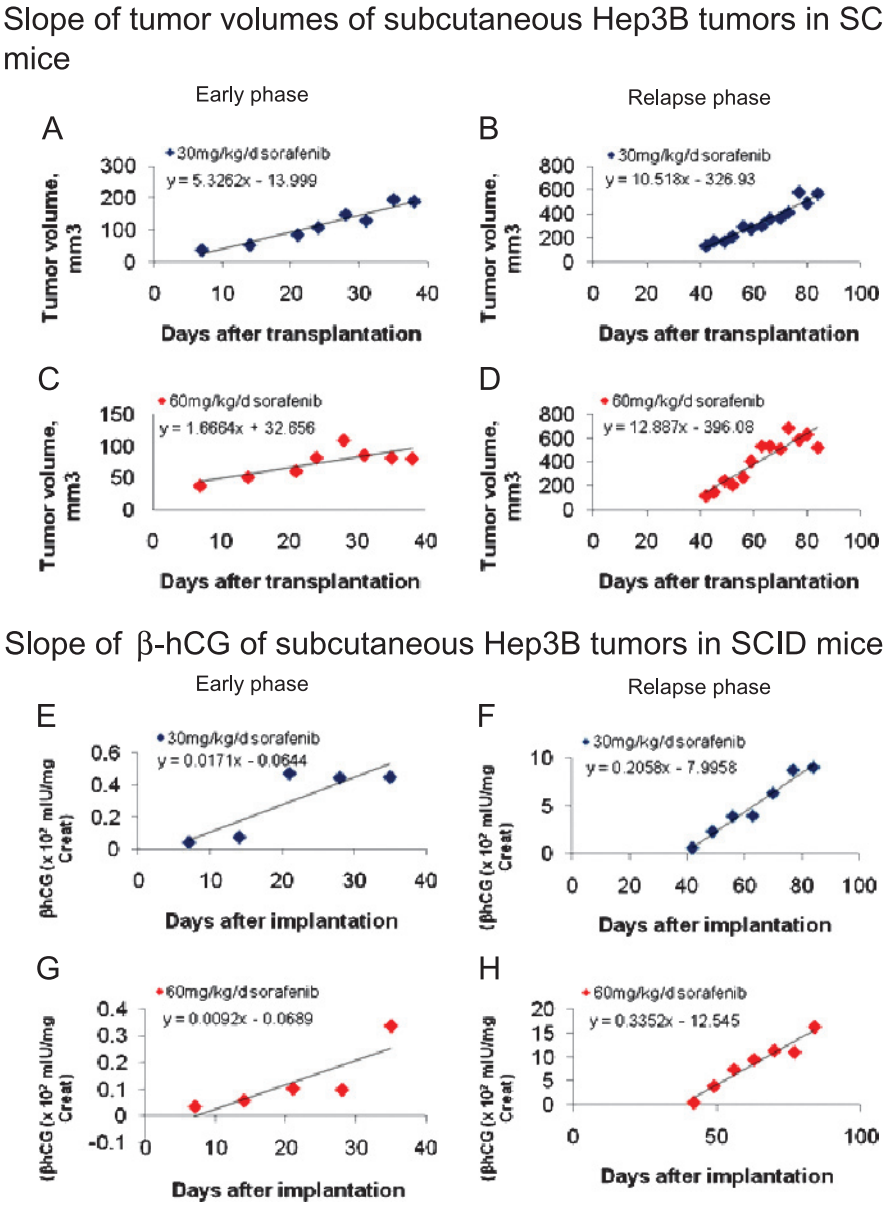


Figure 3. Comparison of slopes of early phase responding tumors and during the later relapse phase of tumor "relapse" of Hep3B subcutaneous tumors in SCID mice. Upper panel shows the slopes of tumor growth curves based on tumor volume measurements. (A) The slope of the early phase tumor volume growth curve from day 7 to 38 in the 30-mg/kg per day of sorafenib-treated mice. (B) Slope of the relapse phase of the tumor volume growth curve, from day 42 to 84 in 30 mg/kg per day of sorafenib-treated mice. (C) Slope of the early phase of the tumor volume growth curve from day 7 to 38 in the 60-mg/kg per day of sorafenib-treated mice. (D) Slope of the relapse phase of the tumor volume growth curve from day 42 to 84 in 60 mg/kg per day of sorafenib-treated mice. Lower panel shows the slopes, based on β-hCG growth curves. (E) Slope of the early phase of the β-hCG curve from day 7 to 35 in the 30-mg/kg per day of sorafenib-treated mice. (F) Slope of the relapse phase of the β-hCG curve from day 42 to 84 in the 30-mg/kg per day sorafenib-treated mice. (G) Slope of the early phase of the β-hCG growth curve from day 7 to 35 in the 60-mg/kg per day of sorafenib-treated mice. (H) Slope of the relapse phase of the β-hCG growth curve from day 42 to 84 in the 60-mg/kg per day of sorafenib-treated mice.

Table 1. Slopes of Tumor Growth Curves of Early and Relapse Phases in the Subcutaneous Hep3B SCID Mice Model.

	Slope between Days 7 and 35 (Early Phase)	Slope between Days 42 and 84 (Relapse Phase)	Fold Change ×
30 mg/kg per day sorafenib (tumor volume)	5.32	10.518	1.97×
60 mg/kg per day sorafenib (tumor volume)	1.66	12.887	7.76×
30 mg/kg per day sorafenib (β-hCG)	0.0171	0.2058	12×
60 mg/kg per day sorafenib (β-hCG)	0.0092	0.3352	36×

as measured by β-hCG levels or tumor volumes, which were maintained for 40 to 50 days of treatment but were then followed by the onset of a more progressive tumor growth—an observation that is generally considered a classic manifestation of (acquired) drug resistance.

An important question is whether the rate of growth when tumors seem to start relapsing on sorafenib therapy is faster than when responding during the first month or so of drug treatment. This would be indicative of onset of tumor "progression" and hence the probable development of resistance. Simple visual inspection of the growth curves in the therapy experiments would seem to indicate that this is so. Nevertheless, we calculated the slopes of the Hep3B tumor growth curves in the subcutaneous transplant model based on both tumor volumes and β-hCG measurements, in drug-treated mice, during the "early" phase of responsive tumor growth and the later "relapse" phase. The results in Figure 3 show steeper slopes indicative of more rapid tumor growth during the later phase, using both types of measurement. Also of interest, the relative changes in growth were assessed to be very similar whether tumor volumes or β-hCG measurements were used for the calculations—another indication that secreted β-hCG can be used as a valid surrogate molecular biomarker of tumor burden/growth. In Table 1, we show the relative slope values and fold changes in growth, which indicate a pronounced increase in tumor growth rates between the initial responding phase and then the "nonresponding" phase in subcutaneous transplant model.

Acquired Resistance-like to Sorafenib Does Not Appear to Be a Heritable Phenotype In Vivo

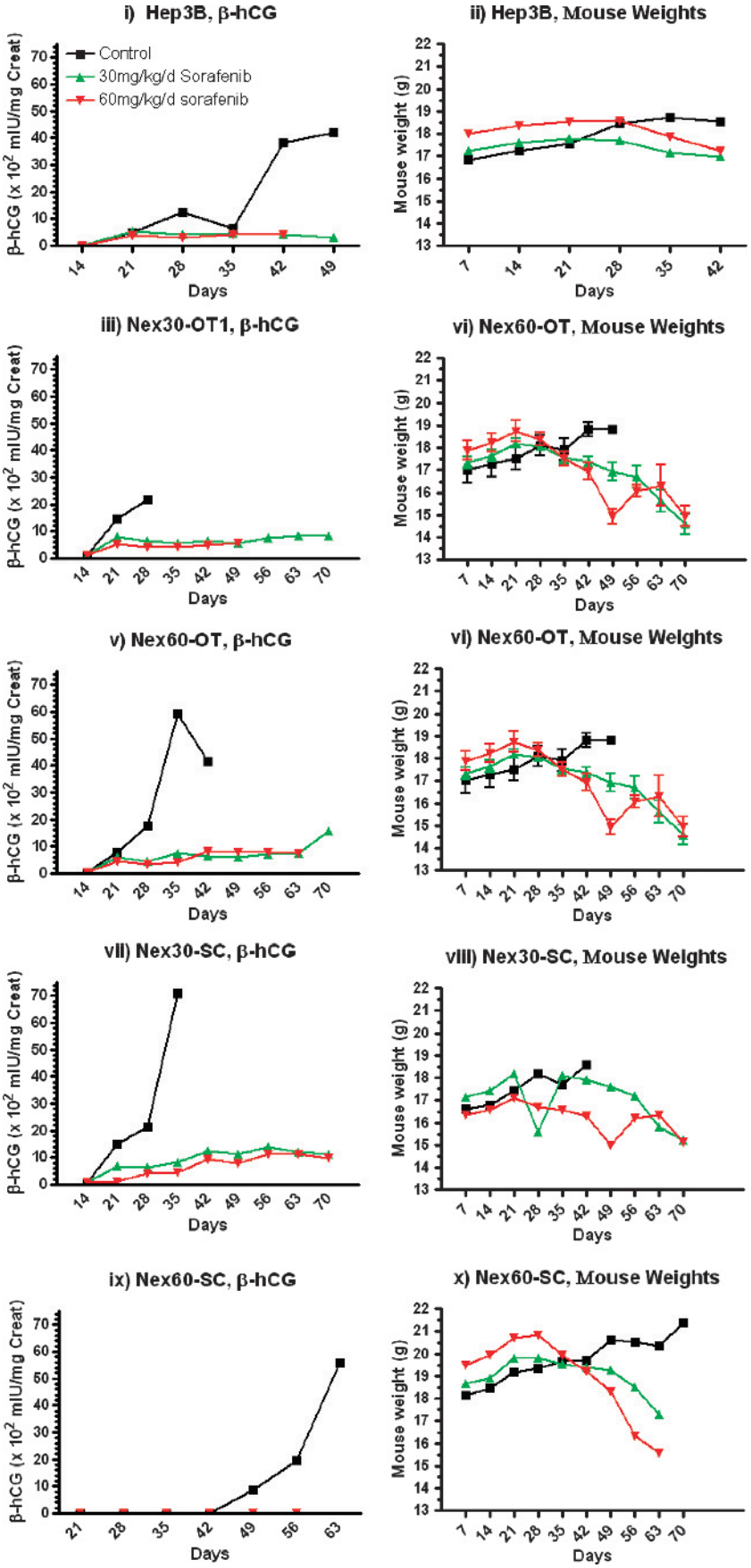
To investigate the nature of the acquired resistance-like to sorafenib that became apparent during the course of our therapy studies, we isolated cells from tumors relapsing on therapy in both our orthotopic and subcutaneous models. Thus, tumors apparently relapsing to sorafenib monotherapy at 30 mg/kg per day on day 85 in the orthotopic model were removed and adapted to tissue culture during 2 weeks, and the resulting cell line was termed *Nex30-OT1*. Similarly, a cell line termed *Nex60-OT* was derived from tumors relapsing on sorafenib monotherapy at 60 mg/kg per day on day 85. From the subcutaneous tumors, Nex30-SC and Nex60-SC cell lines were derived from tumors relapsing on sorafenib monotherapies of 30 and 60 mg/kg per day, respectively. We next asked whether the resistance-like phenotype to sorafenib was heritable. To answer this question, the cell lines from previously mentioned progressing tumors were injected orthotopically into new hosts. We treated these mice by using 30 or 60 mg/kg per day of sorafenib or vehicle control for up to 56 days. We did not detect any evidence of resistance to sorafenib in any of the treatment groups (Figure 4). However, the same toxicity effect observed in earlier experiments was found, that is, weight loss occurred in all treatment groups, and especially severe was the weight loss in the 60-mg/kg per day sorafenib group. Thus, what seems to be acquired resistance to sorafenib did not seem to be stable (propagatable) *in vivo*, when the cells were transferred to new hosts.

Effect of Sorafenib Monotherapy on Orthotopic Hep3B Tumors in Nude Mice

SCID mice seem to be hypersensitive to sorafenib therapy, which hampered long-term administration of the drug. This is not an effect that is restricted to sorafenib as we have observed similar hypersensitivity to other TKIs such as sunitinib. Therefore, we decided to use the same HCC model in nude mice where we speculated toxicity would be less of an issue. Hep3B were implanted into the livers of nude mice, which were then treated 14 days later with control vehicle alone or with sorafenib monotherapy (at 30 or 60 mg/kg per day; Figure 5*A*).

The weight loss observed in the nude mice in this experiment (Figure 5*B*) was less severe than that observed in SCID mice (Figure 1*A*). Nonetheless, some of the nude mice in the 60-mg/kg per day sorafenib treatment group experienced a 10% weight loss at day 84 (i.e., after 70 days of continuous treatment). In addition, one mouse died in the 30-mg/kg per day sorafenib group, and three died in the 60-mg/kg per day group without showing previous signs of overt toxicity (Figure 5*A*). Some of the mice in both treatment groups developed a transient and moderate rash after 1 week of treatment, which was more severe 4 weeks later. The β-hCG levels in the treatment groups (Figure 5*A*) showed a similar pattern to that observed in the HCC model in SCID mice (Figures 1*A* and 2*A*), that is, a pronounced antitumor effect induced by the sorafenib regimens. Thus, sorafenib is effective in inhibiting

Figure 4. Effect of sorafenib regimens on orthotopically implanted Hep3B variants derived from tumors that developed resistance to sorafenib. The cell lines shown in this figure are from a single tumor from each treatment group, and these were implanted into the left lobe of the liver of SCID mice ($n = 5$ per treatment group). The variants tested are (i and ii) Hep3B parental cells, (iii and iv) Nex30-OT1 (a cell line obtained from an orthotopic tumor that relapsed on 30 mg/kg per day sorafenib), (v and vi) Nex60-OT (a cell line obtained from an orthotopic tumor that relapsed on 60 mg/kg per day of sorafenib), (vii and viii) Nex30-SC (a cell line obtained from a subcutaneous tumor that relapsed on 30 mg/kg per day of sorafenib), and (xi and x) Nex60-SC (a cell line obtained from a subcutaneous tumor that relapsed on 30 mg/kg per day of sorafenib). Left panels: Urine hCG levels of pooled samples, normalized to urine creatinine levels, of mice treated with sorafenib monotherapies (at 30 or 60 mg/kg per day by gavage). Compared with control, both sorafenib regimens impaired the growth of the various Hep3B variants comparable to parental Hep3B. Right panels: Corresponding mouse weight curves as a measure of relative toxicity of the different sorafenib regimens.


i) Hep3B, β-hCG
Control
30mg/kg/d Sorafenib
60mg/kg/d sorafenib
β-hCG (x 10² mIU/mg Creat)
Days
ii) Hep3B, Mouse Weights
Mouse weight (g)
Days
iii) Nex30-OT1, β-hCG
vi) Nex60-OT, Mouse Weights
v) Nex60-OT, β-hCG
vi) Nex60-OT, Mouse Weights
vii) Nex30-SC, β-hCG
viii) Nex30-SC, Mouse Weights
ix) Nex60-SC, β-hCG
x) Nex60-SC, Mouse Weights

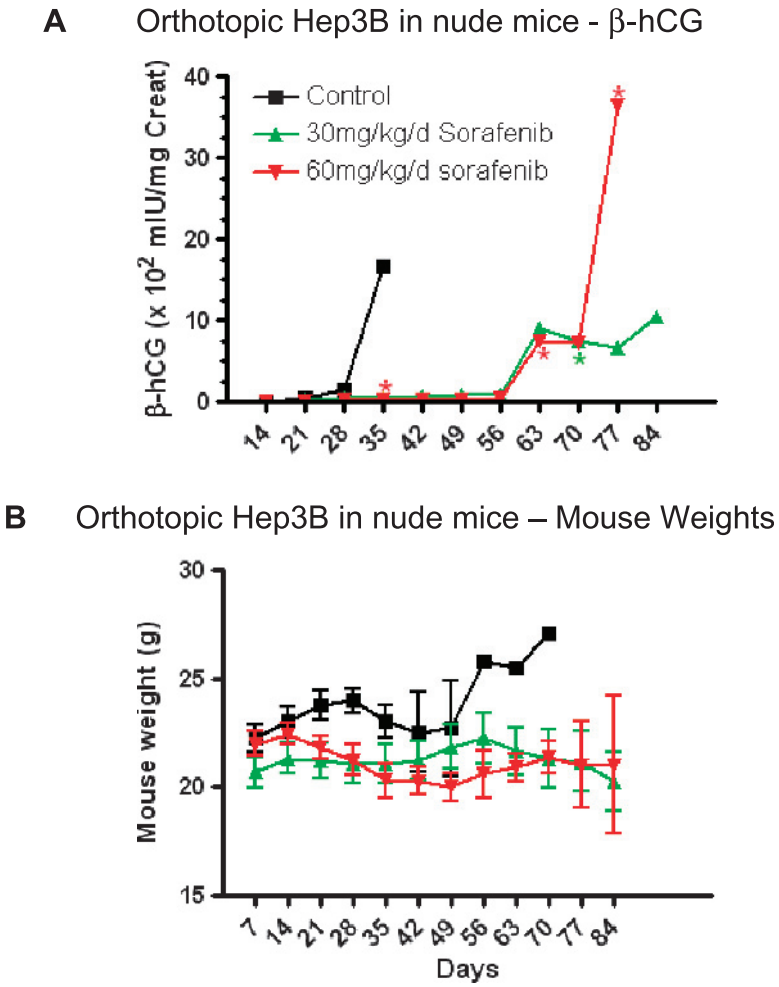


Figure 5. Effect of sorafenib regimens on orthotopically implanted Hep3B-hCG cells in nude mice. Green asterisk indicates a mouse treated with 30 mg/kg per day of sorafenib that died at that time point. Red asterisk indicates a mouse treated with 60 mg/kg per day sorafenib that died at that time point. (A) Urine hCG levels of pooled samples, normalized to urine creatinine levels, of mice treated with sorafenib monotherapies (at 30 or 60 mg/kg per day by gavage). Hep3-hCG tumor growth impairment by sorafenib is similar to the effects seen in SCID mice. (B) However, corresponding mouse weight curves as a measure of relative toxicity reveal a lower degree of toxicity in nude compared with SCID mice.

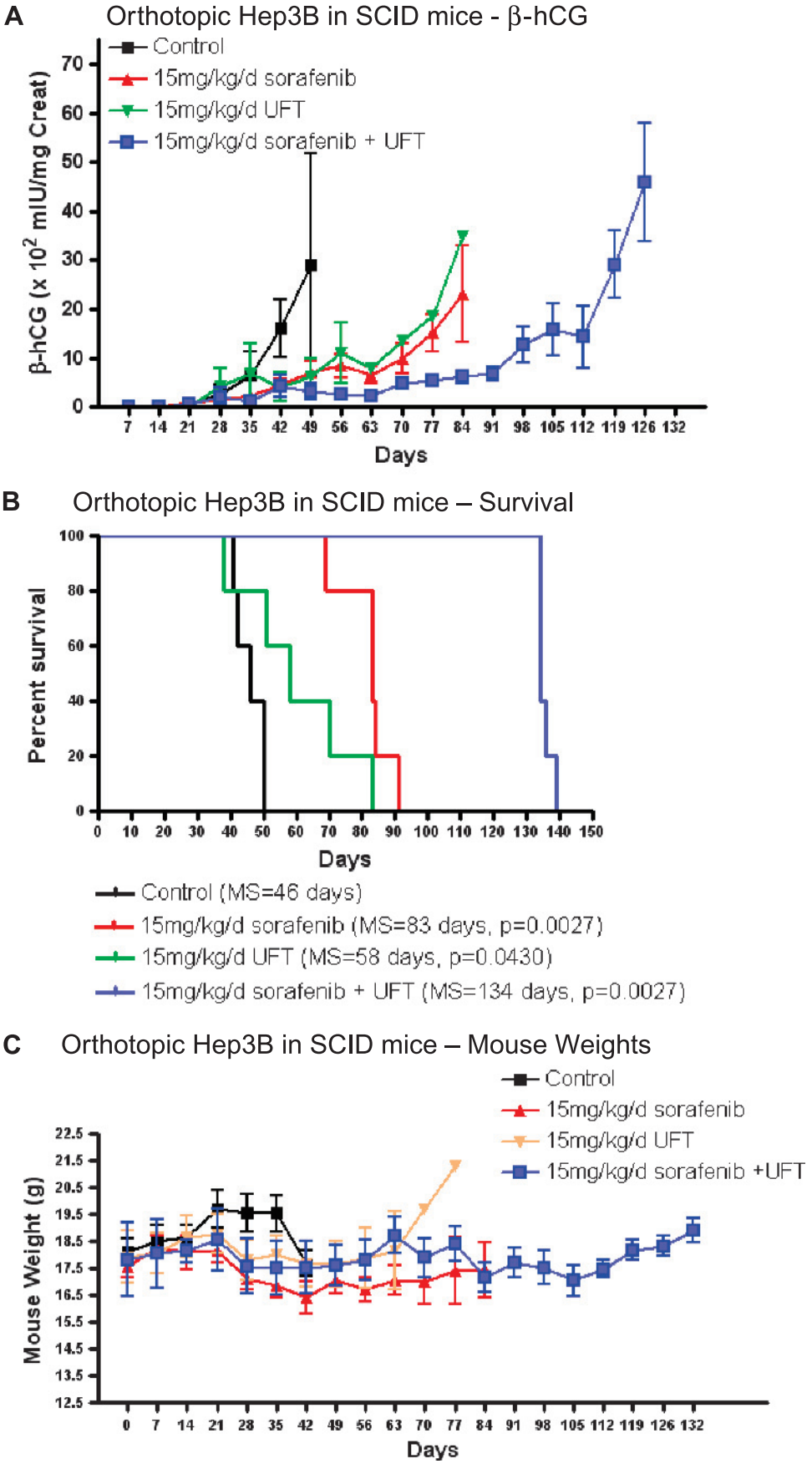
Hep3B tumor growth in both SCID and nude mice, and this benefit is associated with less toxicity in nude mice.

Effect of Combining Sorafenib with Daily Metronomic UFT

We previously evaluated combining sorafenib (at a dose of 30 mg/kg per day) with metronomic UFT (15 mg/kg per day) and observed some increased toxicity (compared with UFT alone—data not shown), after 40 days of therapy with no breaks (Figure 1). Thus, the toxicity seems to be caused by the sorafenib component of the combination treatment. We therefore decided to test a lower dose of sorafenib (15 mg/kg per day) in combination with metronomic UFT chemotherapy. The rationale was that this combination treatment might have a much superior therapeutic ratio, that is, reduced toxicity (thus leading to more extended therapy) accompanied by similar or improved antitumor efficacy. Hep3B cells were implanted orthotopically into SCID mice and then treated with sorafenib or UFT monotherapy (15 mg/kg per day each) or with sorafenib plus metronomic UFT.

The urine β-hCG data curves in this experiment indicated that the combination of sorafenib plus metronomic UFT was highly effective at inhibiting tumor growth (Figure 6*A*), whereas the respective monotherapies were less effective. The benefit of the sorafenib plus metronomic UFT in this model was confirmed by superior median survival

Figure 6. Effect of reduced-dose sorafenib combined with metronomic UFT on orthotopically implanted Hep3B–β-hCG cells in SCID mice (n = 5 per treatment group). (A) Urine β-hCG levels of individual mice, normalized to urine creatinine levels, treated with sorafenib alone (15 mg/kg per day), metronomic UFT alone (15 mg/kg per day), or sorafenib and metronomic UFT combination therapy (15 mg/kg per day each). Although the benefit of the corresponding monotherapies is modest, sorafenib and metronomic UFT combination therapy delays the occurrence of treatment resistance. (B) Kaplan-Meier survival curves confirm the superior treatment benefit of the combination regimen, whereas no significant weight loss is observed. (C) MS indicates median survival.


A
Orthotopic Hep3B in SCID mice - β-hCG
Control
15mg/kg/d sorafenib
15mg/kg/d UFT
15mg/kg/d sorafenib + UFT
β-hCG (x 10² mIU/mg Creat)
Days
B
Orthotopic Hep3B in SCID mice – Survival
Percent survival
Days
Control (MS=46 days)
15mg/kg/d sorafenib (MS=83 days, p=0.0027)
15mg/kg/d UFT (MS=58 days, p=0.0430)
15mg/kg/d sorafenib + UFT (MS=134 days, p=0.0027)
C
Orthotopic Hep3B in SCID mice – Mouse Weights
Control
15mg/kg/d sorafenib
15mg/kg/d UFT
15mg/kg/d sorafenib +UFT
Mouse Weight (g)
Days

(Figure 6*B*), which, importantly, was not compromised by any significant host toxicity associated with the combination treatment regimen (Figure 6*C*). Thus, of all the treatments tested in this series of studies, the metronomic UFT/sorafenib combination seemed to be the most effective in terms of both efficacy and (reduced) toxicity.

Discussion

The increasing marketing approvals and clinical uses of antiangiogenic drugs such as bevacizumab, sorafenib, sunitinib, and pazopanib highlight the need for developing preclinical models designed to study the basis of intrinsic or acquired resistance to such agents, both of which are common occurrences in patients which limit their efficacy [1–3]. Although a number of such preclinical resistance models have been described for antibody or other protein-based antiangiogenic drugs (see Introduction), only two currently exist for the oral small-molecule antiangiogenic TKIs, and both involve sunitinib and renal cell carcinoma [16,17]. Hence, our study adds to this small but important body of literature and, moreover, is the first to deal with sorafenib and HCC. In this regard, it is noteworthy that our results indicate what seems to be a reversible (i.e., nonpropagatable) form of resistance-like phenotype to sorafenib. Our results seem to be a classic manifestation of the development of drug resistance on therapy, in which treated tumors show an initial robust tumor response and then "progressive disease" at a later time point. Detecting this response was made possible, or facilitated, by three important technical considerations: 1) an HCC tumor line (Hep3B) that is highly sensitive, at least initially, for over a month to sorafenib therapy; 2) the use of a surrogate molecular marker, namely, secreted β-hCG to monitor tumor growth, response, and then progression over time in the orthotopic model; and 3) the application of extended therapy regimens lasting at least until evidence of a tumor relapse-like effect becomes evident with more rapid resumption of tumor growth. Below we discuss some of the critical aspects, including caveats, of the models we developed, the results obtained, as well as some of the clinical implications of the results.

Effect of Sorafenib Treatment Interruptions

Because of the rapid *in vivo* growth of the Hep3B model [20], we initiated treatment as soon as the disease was detectable by urine hCG levels. A possible caveat of our models was the necessity to interrupt the daily sorafenib treatments using the 30- or 60-mg/kg doses because of toxicity (as manifested by loss of body weight ≥10%). However, such interruptions are not uncommon in the clinic, sometimes occurring in as many as 30% to 50% of metastatic cancer patients receiving sorafenib or other similar TKI drugs [19,32–35]. Thus, in a sense, our models, some of which also involve orthotopic tumor transplants, mimic the clinical situation in a more faithful way than only using continuous (and very short term) treatments of only subcutaneous tumor model transplants. Nevertheless, future preclinical experiments designed to investigate in greater depth the effect of discontinuous *ver*sus continuous treatment regimens on outcomes, including drug resistance, would seem warranted.

With respect to the toxicities encountered, it would seem that SCID mice are especially sensitive to the toxicity of the sorafenib treatments. This is not a sorafenib specific effect as we have observed similar results with a number of other TKIs such as sunitinib (Ebos et al., unpublished observations). SCID mice, because of a mutational defect of DNA-dependent protein kinase, are known to be hypersensitive to certain anticancer treatments involving DNA damage such as radiation [36]. The basis for the apparent hypersensitivity of SCID mice to TKI drugs such as sorafenib, sunitinib, but apparently not others such as lapatinib [37], remains to be determined.

Apparent Reversibility of the Phenotype of Acquired Sorafenib Resistance

The experiments designed to evaluate the stability of the apparent resistance to sorafenib, which developed after at least 1 month of daily therapy (with a short interruption during the therapy), indicate an apparent reversibility of the phenotype, as assessed by retransplantation and treatment of the tumor cells in new hosts. One technical issue about this experiment is that the tumors relapsing on sorafenib therapy in the original treatment host were removed and cultured for at least 2 weeks, after which the cells were injected into new hosts, and then sorafenib treatment initiated after approximately 2 weeks (on the established primary tumors). This means that the "resistant" cells were not exposed to sorafenib for about 1 month before they were exposed to treatment again. It may be that continuous exposure to drug is necessary to maintain the resistant phenotype [38]. If so, treatment interruptions might actually slow the development of resistance. One important implication of this possibility is that tumors in patients relapsing on sorafenib might respond again to the same drug after a defined break period, provided, of course, that progression of such tumors is relatively slow. There is some preliminary evidence that RCC tumors in patients who stop responding to sorafenib may respond to a later treatment of a similar drug, for example, sunitinib [2,4,5]. On the basis of our results, it may be that these patients might have also responded to sorafenib again. These speculations simply reinforce the need to study the apparent reversible nature of the sorafenib drug resistance phenotype that may occur in some instances and the effect of treatment interruptions on the kinetics of resistance and (re)response. In this regard, we would note the recent report by Hammers et al. [16] who found that xenografts established from a biopsy of an RCC patient who had progressed on sunitinib after showing an initial response, regained sensitivity to the drug, and that this was associated with a reversible epithelial-to-mesenchymal phenotypic transition. Thus, our results are operationally and conceptually similar to those of Hammers et al., despite using HCC cell lines and a different drug.

Implications of Addition of Concurrent Metronomic Chemotherapy

The kinetics of the resistance to sorafenib in our model facilitated an experimental analysis of one possible strategy to delay the onset of such resistance. Our preliminary results using a lower, less toxic, dose of sorafenib (15 mg/kg) with metronomic UFT are encouraging in this regard because the combination provided the best therapeutic results of all the various treatments we assessed and, moreover, did so in the absence of any overt toxicity. This result, although preliminary, deserves further investigation because sorafenib treatments at current recommended doses can cause a plethora of toxic adverse effects in patients [39,40], which, as already discussed, can lead to interruptions in therapy and/or reductions in drug dose [19,32–35]. If the dose of sorafenib can be lowered by combination with another drug treatment known to be minimally or nontoxic in the clinic, for example, metronomic UFT, which has been used in a daily dosing (and uninterrupted) schedule for 2 years in adjuvant treatment settings [28,41], such a combination protocol would seem ideal for clinical evaluation with sorafenib. This could be particularly worthwhile for the postoperative adjuvant treatment of RCC or HCC. An additional rationale for this consideration, aside

from the precedents of randomized phase 3 clinical trial successes of adjuvant metronomic UFT in non–small cell lung cancer [28] and breast cancer [41] is that the addition of the metronomic chemotherapy may prevent the possible tumor growth promoting effects of antiangiogenic TKIs in adjuvant-like treatment settings that were recently described by us in preclinical studies [42].

The decision to emphasize UFT for metronomic chemotherapy for HCC in our studies with sorafenib might be questioned in view of some clinical results showing UFT does not have activity in advanced HCC [43]. However, there are other reports indicating UFT may have such activity in advanced HCC [44,45]. Furthermore, an antitumor effect of UFT may become more evident when it is combined with another drug such as sorafenib, especially in the postoperative adjuvant setting of HCC where low tumor volumes are present [46]. Indeed, although these preclinical studies were being completed, a clinical report of a phase 2 trial involving treatment of HCC patients with sorafenib and metronomic UFT was published, showing encouraging results, in terms of both clinical benefit and safety/tolerability [47].

In summary, our results suggest that the development of a resistant-like phenotype in HCC cells to sorafenib seems to be reversible. Although the mechanistic basis of this reversible phenotype is unknown, it is likely to result from the fact that sorafenib is mainly targeting a host process—tumor angiogenesis—rather than directly directing the tumor cell population. The reversible phenotype has a number of implications in the clinic, as we have discussed; finally, we have outlined a possible strategy, based on preliminary results, that seems effective in delaying the development of resistance to sorafenib, but without added toxicity—concurrent metronomic chemotherapy, in this case using oral UFT.

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